Reductive Formation of Carbon Monoxide from CCl_4 and FREONs 11, 12, and 13 Catalyzed by Corrinoids†

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ABSTRACT: In an earlier publication, we reported that corrinoids catalyze the sequential reduction of CCl_4 to $CHCl_3$, CH_2Cl_2, CH_3Cl, and CH_4 with titanium(III) citrate as electron donor [Krone, U. E., Thauer, R. K., & Hogenkamp, H. P. C. (1989) *Biochemistry 28*, 4908–4914]. However, the recovery of these products was less than 50%, indicating that other products were formed. We now report that, under the same experimental conditions, CCl_4 is also converted to carbon monoxide. These studies were extended to include FREONs 11, 12, 13, and 14. Corrinoids were found to catalyze the reduction of $CFCl_3$, CF_2Cl_2, and CF_3Cl to CO and, in the case of $CFCl_3$, to a lesser extent, to formate. CF_4 was not reduced. The rate of CO and formate formation paralleled that of fluoride release. Both rates decreased in the series $CFCl_3$, CF_2Cl_2, CCl_4, and CF_3Cl. The reduction of $CFCl_3$ gave, in addition to CO and formate, $CHFCl_2$, CH_2FCl, CH_3F, $C_2F_2Cl_2$, and $C_2F_2Cl_4$. The product pattern indicates that the corrinoid-mediated reduction of halogenated C_1-hydrocarbons involves the intermediacy of dihalocarbenes, which may be a reason why these compounds are highly toxic for anaerobic bacteria.

Many anaerobic eubacteria and archaebacteria are able to degrade CCl_4 to $CHCl_3$, CH_2Cl_2, CH_3Cl, and CO_2, indicating reductive and hydrolytic conversions [for recent literature, see Egli et al. (1990) and Gälli and McCarty (1989)].

$$CCl_4 + 2[H] \rightarrow CHCl_3 + HCl \quad E^{\circ\prime} = +0.516\ V \quad (a)$$

$$CCl_4 + 4H_2O \rightarrow CO_2 + 4HCl \quad \Delta G^{\circ\prime} = -65\ kJ/mol \quad (b)$$

(Wagman et al., 1968) (Thermodynamic data were calculated from the free energies of formation: CCl_4 and $CHCl_3$ in the liquid state; CO_2 in the gaseous state; 1 M Cl^- in aqueous solution; pH 7.) The anaerobes known to mediate these dehalogenation reactions have in common that they use the acetyl-CoA/carbon monoxide dehydrogenase pathway for the degradation and synthesis of acetyl-CoA. The key step in this pathway is the reversible transfer of the methyl group of acetyl-CoA to a tetrahydropterin (H_4P), either tetrahydrofolate or tetrahydromethanopterin, yielding CH_3-H_4P, CO_2, and two reducing equivalents ([H]) (Wood et al., 1986a,b; Thauer et al., 1989; Thauer, 1990).

$$CH_3CO\text{-}SCoA + H_4P \rightarrow CH_3\text{-}H_4P + CO_2 + 2[H] + CoASH \quad (c)$$

$$\Delta G^{\circ\prime} = +41.3\ kJ/mol$$

This methyl transfer proceeds via the methylated corrinoid protein $[Co^I]E$[1] as the intermediate (Ragsdale et al., 1987; Lu et al., 1990).

$$CH_3CO\text{-}SCoA + [Co^I]E \rightarrow CH_3\text{-}[Co^{III}]E + CO_2 + 2[H] + CoASH \quad (d)$$

$$CH_3\text{-}[Co^{III}]E + H_4P \rightarrow CH_3\text{-}H_4P + [Co^I]E \quad (e)$$

Reaction d is mediated by the nickel–protein carbon monoxide dehydrogenase (Wood et al., 1986a,b; Shanmugasundaram et al., 1989; Lindahl et al., 1990a,b), designated as such because it also catalyzes the dehydrogenation of CO + H_2O to CO_2 and 2[H]. Reaction e is catalyzed by a methyltransferase (Wood et al., 1986a,b).

It has been suggested that the corrinoid protein $[Co^I]E$ may mediate the dehalogenation of chlorinated C_1-hydrocarbons in anaerobic bacteria. This proposal is based on the observation that all bacteria that are able to catalyze the dehalogenation reactions contain this corrinoid protein (Egli et al., 1990) and on our earlier findings that free corrinoids catalyze the reductive dehalogenation of CCl_4 to $CHCl_3$, CH_2Cl_2, CH_3Cl, and CH_4 with titanium(III) citrate or dithiols as electron donors (Krone et al., 1989b). In methanogenic bacteria, corrinoids and coenzyme F_{430} may be involved as catalysts (Krone et al., 1989a).

The reduction of CCl_4 to $CHCl_3$ (reaction a) catalyzed by corrinoids ([Co]) may involve trichloromethyl corrinoids as intermediates (reactions f and g or h).

$$CCl_4 + [Co^I] \rightarrow Cl^- + CCl_3[Co^{III}] \quad (f)$$

$$CCl_3[Co^{III}] + e^- + H^+ \rightarrow CHCl_3 + [Co^{II}] \quad (g)$$

$$CCl_3[Co^{III}] + 2e^- + H^+ \rightarrow CHCl_3 + [Co^I] \quad (h)$$

A one- or two-electron reduction of the latter would generate $CHCl_3$ and a Co(II) or a Co(I) corrinoid (Krone et al., 1989b). Egli and co-workers (1988, 1990) have shown that some

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[1] Abbreviations: FREON 11, $CFCl_3$; FREON 12, CF_2Cl_2; FREON 13, CF_3Cl; FREON 14, CF_4; Cbl, cobalamin; [Co], corrinoid.

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anaerobic bacteria are able to degrade CCl_4 and $CHCl_3$ to CH_2Cl_2, CH_3Cl, and CO_2. The production of CO_2 was surprising because it was not one of the products of the reductive dehalogenation of either CCl_4 or $CHCl_3$ catalyzed by corrinoids.

It has been well established that cytochrome P_{450} can also catalyze the reductive dehalogenation of many halogenated aliphatic hydrocarbons (Ruf et al., 1984, and references quoted therein). During these reactions, complexes of cytochrome P_{450} with iron–carbon bonds are formed as intermediates. For instance, the reductive dehalogenation of CCl_4 generates a ferrous carbene complex $[(P_{450})Fe^{II}(CCl_2)]$ that slowly hydrolyzes to CO.

The present paper demonstrates that CO is also a product of the reductive dehalogenation of CCl_4 by titanium(III) citrate catalyzed by the corrinoids and thus provides the basis for the CO_2 production observed by Egli et al. (1988, 1990). In this series of reactions, CCl_4 is reduced to dichlorocarbene (reaction i), which hydrolyzes to CO (reaction j) or formate (reaction k) (Hine, 1950). Carbon monoxide and formate are then oxidized to CO_2 via the very active CO dehydrogenase (reaction l) or formate dehydrogenase (reaction m) present in the anaerobic bacteria (Wood et al., 1986a,b; Thauer et al., 1977, 1989).

$$CCl_4 + 2[H] \rightarrow CCl_2 + 2HCl \quad \text{(i)}$$

$$CCl_2 + H_2O \rightarrow CO + 2HCl \quad \text{(j)}$$

$$CCl_2 + 2H_2O \rightarrow HCOOH + 2HCl \quad \text{(k)}$$

$$CO + H_2O \rightarrow CO_2 + 2[H] \quad E^{\circ\prime} = -524 \text{ mV} \quad \text{(l)}$$

$$HCOO^- + H^+ \rightarrow CO_2 + 2[H] \quad E^{\circ\prime} = -432 \text{ mV} \quad \text{(m)}$$

Recently, Dolbier and Burkholder (1988, 1990) showed that the reduction of $CFCl_3$ by metals generates fluorochlorocarbene with a high efficiency. These reports prompted us to also investigate the reductive dehalogenation of FREONs 11 (FCl_3C), 12 (F_2Cl_2C), and 13 (F_3ClC) to the corresponding dihalocarbenes by corrinoids.

Materials and Methods

Carbon tetrachloride, methylene chloride, trichlorofluoromethane, and titanium(III) chloride were purchased from Merck, chloroform was from Baker Chemicals, methyl chloride and methane were from Messer Griesheim, dichlorodifluoromethane, chlorotrifluoromethane and tetrafluoromethane were from Hoechst, and bromotrifluoromethane, chlorodifluoromethane, and dichlorofluoromethane were from Union Carbide. Cyanocobalamin, hydroxocobalamin hydrochloride (aquocobalamin), and methylcobalamin were obtained from Sigma Chemical Co. All other reagents and chemicals were commercial products of the highest purity and were used as received. Titanium(III) citrate solutions (0.078 M) were prepared as described by Zehnder and Wuhrmann (1976), except that the pH was adjusted to 8 with 2 M Tris-HCl buffer rather than with Na_2CO_3. Standard solutions of $CHCl_3$ (0.074 M) and CH_2Cl_2 (0.093 M) were prepared by dissolving 0.3 mL of the chlorinated hydrocarbon in 50 mL of methanol; standard solutions of CCl_4 (0.312 M) and $CFCl_3$ (0.326 M) were obtained by dissolving 1.5 mL in 50 mL of methanol. The gaseous standards CH_3Cl and CH_4 were prepared by injecting 0.25 mL of the gas into a 120-mL serum bottle closed with a viton stopper, containing N_2 at 1.4×10^5 Pa; 0.3 mL of these mixtures contains 20 nmol of the gas. Carbon dioxide, CO, $CFCl_3$, CCl_2F_2, $CClF_3$, and CF_4 were standardized by the headspace method (Schwedt, 1979). (Cyanoaquo)cobinamide and (cyanoaquo)-3,5,6-trimethylbenzimidazoylcobamide were prepared from cyanocobalamin as described by Friedrich and Bernhauer (1956).

The fluoromethylcobalamins were prepared by a modification of the procedure described by Dolphin (1971). Aquocobalamin (1.0 g), dissolved in a deaerated 10% w/v aqueous ammonium chloride solution (80 mL) containing 10 mL of ethanol, was reduced with zinc dust (8 g). After about 20 min, the solution was filtered under argon to remove the excess zinc, the gray-green filtrate was reacted with 300 mL of the appropriate gaseous fluorocarbon (BrF_3C, ClF_2HC, Cl_2FCH), and the reaction was stirred vigorously for 30 min. The reaction mixtures were desalted by phenol extraction and the concentrated aqueous solutions chromatographed on SP-Sephadex. The desired fluoromethylcobalamins were eluted with water, concentrated, and crystallized from aqueous acetone. The purity of the fluoromethylcobalamins was established by high-pressure liquid chromatography on a Brownlee Labs Aquapore R.P. 300 C-8 column with the gradient elution method described by Jacobsen et al. (1986). Monochloromethylcobalamin was prepared from cob(I)alamin and methylene chloride, as described before (Hogenkamp et al., 1965). The authenticity of the fluoromethylcobalamins was established by ^{19}F nuclear magnetic resonance spectroscopy with a General Electric Omega 500-MHz spectrometer (Brown et al., 1984). ^{19}F NMR spectra of CFH_2Cbl, CF_2HCbl, and CF_3Cbl in D_2O showed a triplet at −50.3 ppm (J_{H-F} = 44.5 Hz), a doublet at 72.0 ppm (J_{H-F} = 56.3 Hz), and a singlet at 135.4 ppm (from neat external hexafluorobenzene), respectively. The splitting of the fluorine resonance of CFH_2Cbl into a triplet demonstrates that the expected product from the reaction between cob(I)alamin and Cl_2FCH, CFClHCbl, was further dechlorinated during the reaction. Ultraviolet–visible spectra were recorded on a Gilford response recording spectrophotometer or on a Hewlett-Packard (model 8452A) diode array spectrophotometer. The concentrations of the corrinoid solutions were determined by using the following extinction coefficients: aquocobalamin, $\epsilon_{527} = 8.5 \times 10^3$ M^{-1} cm^{-1}; methylcobalamin, $\epsilon_{528} = 7.9 \times 10^3$ M^{-1} cm^{-1}; (cyanoaquo)cobinamide, $\epsilon_{527} = 8.4 \times 10^3$ M^{-1} cm^{-1}; (cyanoaquo)trimethylbenzimidazoylcobamide, $\epsilon_{527} = 8.4 \times 10^3$ M^{-1} cm^{-1}; (methylaquo)cobinamide, $\epsilon_{462} = 10.7 \times 10^3$ M^{-1} cm^{-1} (Friedrich, 1975); trifluoromethylcobalamin, $\epsilon_{540} = 9.9 \times 10^3$ M^{-1} cm^{-1}; difluoromethylcobalamin, $\epsilon_{520} = 10.0 \times 10^3$ M^{-1} cm^{-1}; fluoromethylcobalamin, $\epsilon_{518} = 9.3 \times 10^3$ M^{-1} cm^{-1}; chloromethylcobalamin, $\epsilon_{522} = 9.6 \times 10^3$ M^{-1} cm^{-1}.

All dehalogenation experiments were carried out in 120-mL serum bottles that were closed with viton stoppers and wrapped in aluminum foil. The anaerobic reaction components were injected into the serum bottles that were first rigorously deaerated and then filled with N_2 at 1.4×10^5 Pa pressure. The reaction mixtures (30 mL) were incubated at 30 °C in a shaking water bath (New Brunswick Scientific). At the desired time intervals, 0.3-mL gas samples were withdrawn with a gas-tight syringe and analyzed by gas chromatography. For fluoride determination, 1-mL samples of the reaction mixture were withdrawn, and the reaction was stopped by the addition of 4 mL of 0.3% H_2O_2 in 0.2 M Tris-HCl buffer, pH 7.5.

The chlorinated and fluorinated hydrocarbons and methane were separated by using a Carlo Erba Strumentazione (GC 6000) gas chromatograph containing a column (6 mm × 1.8 m) of 80/120 carbopack B-DA/4% carbowax (Supelco). The gas chromatograph was equipped with a flame-ionization detector. The conditions were as follows: column temperature, 110 °C; injector temperature, 160 °C; detector temperature,

Table I: Halogenated C_1-Compounds Formed from $CFCl_3$ by the Corrinoid-Catalyzed Reduction with Titanium(III) Citrate as Electron Donor[a]

time (min)	$CFCl_3$ (units)	$CHFCl_2$ (units)	CH_2FCl (units)	CH_3F (units)	$C_2F_2Cl_2$ (units)	$C_2F_2Cl_4$ (units)
0	60	0	0	0	0	0
15	32.2	3.1	0.4	0.4	0	0
30	9.5	4.4	0.5	0.5	0.6	0
45	1.8	4.3	0.5	0.5	2.7	traces
60	0	3.8	1.0	0.6	4.5	traces

[a] The same experiment as described in Figure 2. The products were identified by mass spectrometry after separation by gas chromatography. Quantitation was by flame-ionization detection. One unit is the amount of product yielding the same ionization signal as 1 μmol of $CFCl_3$ in the assay. An exact calibration was not possible because reference substances for the products were not available.

160 °C; N_2 flow, 60 mL/min; H_2 flow, 35 mL/min; air flow, 240 mL/min. Retention times were as follows: CH_4, 31 s; CH_3F, 37 s; CF_3Cl, 40 s; CH_3Cl, 53 s; CH_2FCl, 63 s; CF_2Cl_2, 65 s; CH_3OH, 95 s; $CHFCl_2$, 133 s; $C_2F_2Cl_2$, 149 s; $CFCl_3$, 160 s; CH_2Cl_2, 164 s; $CHCl_3$, 406 s; CCl_4, 533 s. CO_2 and CO were determined with the same gas chromatograph with a methanizer at 375 °C and a column (2 mm × 2 m) of 60/80 carbosieve B (Supelco); the retention times were 60 s for CO and 270 s for CO_2. $CHFCl_2$, CH_2FCl, CH_3F, $C_2F_2Cl_2$, and $C_2F_2Cl_4$ were identified by gas chromatography/mass spectrometry with a TRIO-2 GC/MS combination (VG Instruments, Altrincham) by using a 30 m × 0.25 mm DB-1 capillary column (J & W Scientific). The column temperature was 30 °C and was increased to 150 °C (10 °C/min) 5 min after the injection. The pressure of the carrier gas (He) was 3.5×10^5 Pa. Formate was concentrated by liquid–liquid extraction of the acidified reaction mixture with diethyl ether and was determined enzymatically with a NAD-dependent formate dehydrogenase (Boehringer Mannheim) as outlined in *Methoden der Enzymatischen Lebensmittelanalytik* (1984). Fluoride was measured with a fluoride electrode (Ingold Steinbach) by using a silver/silver chloride electrode as reference electrode and a type PHM 64 research pH meter (Radiometer) as potentiometer. The electrode was calibrated with NaF in 0.2 M Tris-HCl, pH 7.5.

Results

Carbon Monoxide Formation from CCl_4. Aquocobalamin catalyzed the reductive dehalogenation of CCl_4 to CO with titanium(III) citrate as the reducing agent. The results of a typical experiment are presented in Figure 1. Under the experimental conditions, approximately 6% of the CCl_4 was converted to CO, 54% to $CHCl_3$, 13% to CH_2Cl_2, and 1.5% to CH_3Cl. No CO_2 could be detected after acidification of the reaction mixture. The formation of CO, like that of the other products, was dependent on the presence of the corrinoid. In its absence, no CO and only traces of $CHCl_3$ were formed. Since the carbon in $CHCl_3$ is at the same oxidation level as in CO, it is possible that CO formation from CCl_4 proceeded via $CHCl_3$ as the intermediate. Indeed, $CHCl_3$ was converted to CO in the presence of titanium(III) citrate and aquocobalamin. However, the rate of CO formation from $CHCl_3$ (0.75 μmol in 60 min) was only a fraction of that of CO formation from CCl_4, indicating that free $CHCl_3$ is not an intermediate. In this context, it is of interest that *Methanosarcina* strains catalyze the conversion of $CHCl_3$ to CO_2 (Mikesell & Boyd, 1990), suggesting a corrinoid-catalyzed CO production, followed by its oxidation by CO dehydrogenase (Bott & Thauer, 1989).

Reductive Dehalogenation of $CFCl_3$. FREON 11 was reduced by titanium(III) citrate in the presence of aquocobalamin as a catalyst (Figure 2). The major product was CO (67%); $CHFCl_2$ (<10%), formate (<5%), and lesser amounts of CH_2FCl, CH_3F, $C_2F_2Cl_2$, and $C_2F_2Cl_4$ were also formed (Table I). The recovery of identified products was 90%. No

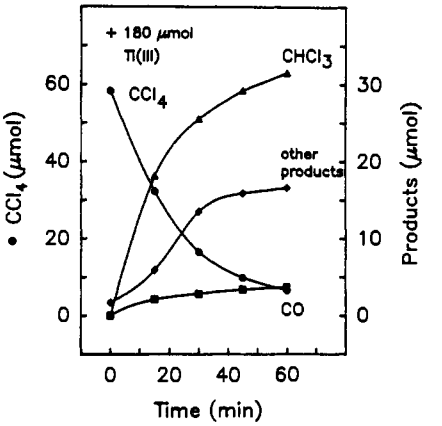


FIGURE 1: Corrinoid-catalyzed reductive dehalogenation of CCl_4 with titanium(III) citrate as electron donor. The assays were performed in the dark at 30 °C in 120-mL serum bottles with N_2 as the gas phase. The 30-mL reaction mixture contained 60 μmol of CCl_4, 180 μmol of titanium(III) citrate, and 1.5 μmol of aquocobalamin in 0.2 M Tris-HCl, pH 7.5. The reaction was started by the addition of cobalamin. At the indicated intervals, 0.3-mL samples of the headspace were analyzed by gas chromatography. "Other products" were CH_2Cl_2, CH_3Cl, and CH_4.

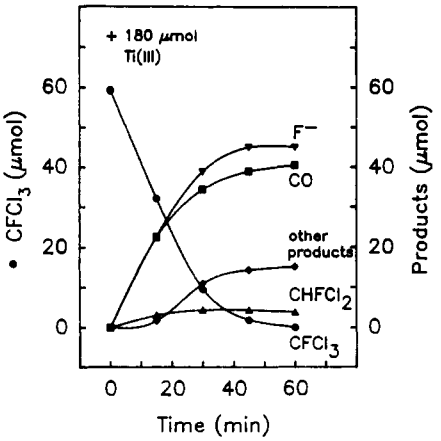


FIGURE 2: Corrinoid-catalyzed reductive dehalogenation of $CFCl_3$ with titanium(III) citrate as electron donor. The assays were performed in the dark at 30 °C in 120-mL serum bottles with N_2 as the gas phase. The 30-mL reaction mixture contained 60 μmol of $CFCl_3$, 180 μmol of titanium(III) citrate, and 1.5 μmol of aquocobalamin in 0.2 M Tris-HCl, pH 7.5. The reaction was started by the addition of cobalamin. At the indicated intervals, 0.3-mL samples of the headspace were analyzed by gas chromatography and 1-mL samples of the liquid phase were withdrawn and analyzed for fluoride. For "other products", see Table I.

reduction was observed in the absence of aquocobalamin. The rate and extent of fluoride release was virtually identical with that of the sum of CO and formate formation (Figure 2). Neither CH_4 nor CO_2 could be detected. The rates of conversion to the various products were almost the same for $CFCl_3$ and CCl_4 (Figures 1 and 2). However, the extent of CO formation from $CFCl_3$ was at least 10 times higher than that from CCl_4. In contrast, the extent of $CHFCl_2$ formation from

Table II: Reduction of $CFCl_3$ with Titanium(III) Citrate Catalyzed by Different Corrinoids[a]

corrinoid	$CFCl_3$[b] (μmol)	CO[b] (μmol)	F^-[b] (μmol)	$CHFCl_2$[b] (μmol)
aquocobalamin	2.5	29.3	42.0	9.8
(cyanoaquo)cobinamide	0.7	32.5	45.0	8.2
(methylaquo)cobinamide	1.1	31.7	45.0	10.3
(cyanoaquo)trimethylbenzimidazoylcobamide	0.7	31.7	46.5	9.1
methylcobalamin	12.7	18.3	30.0	7.2
monofluoromethylcobalamin	7.5	19.5	39.0	6.9
difluoromethylcobalamin	5.2	22.3	31.5	7.7
trifluoromethylcobalamin	1.8	25.2	45.0	8.8

[a] For assay conditions, see Figure 2. The assay mixture contained 60 μmol of $CFCl_3$, 180 μmol of titanium(III) citrate, and 1.5 μmol of the indicated corrinoid. [b] After 60 min.

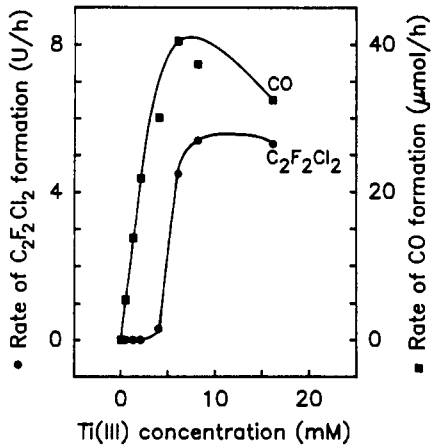


FIGURE 3: Dependence of the rates of CO formation and $C_2F_2Cl_2$ formation from $CFCl_3$ on the titanium(III) citrate concentration. The assay conditions were those described in Figure 2, except that the titanium(III) citrate concentration was varied as indicated. The amount of $C_2F_2Cl_2$ formed is given in units. One unit is the amount yielding the same flame ionization signal as 1 μmol of $CFCl_3$ in the assay (see Table I).

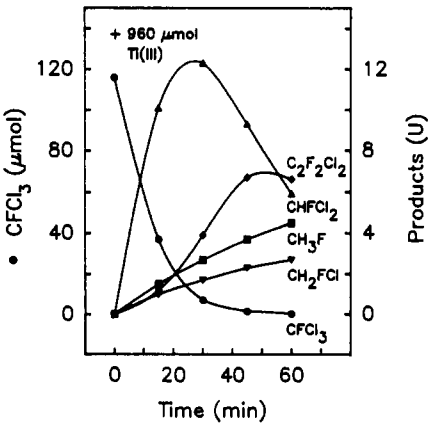


FIGURE 4: Corrinoid-catalyzed reductive dehalogenation of $CFCl_3$ with titanium(III) citrate as electron donor. The experiment differs from that described in Figure 2 in that the molar ratio of Ti(III)/$CFCl_3$ was increased from 3/1 to 8/1 and that the amount of aquocobalamin was twice as high (3 μmol). The amounts of halogenated products are given in units. One unit is the amount yielding the same flame ionization signal at 1 μmol of $CFCl_3$ in the assay (see Table I).

$CFCl_3$ was much lower than that of $CHCl_3$ formation from CCl_4 (Figures 1 and 2).

In the experiments described in Figures 1 and 2 and in Table I, 180 μmol of titanium(III) citrate was used to reduce 60 μmol of CCl_4 or $CFCl_3$. Thus, only a 50% excess in reducing equivalents required for a two-electron reduction was available. When the Ti(III) concentration was increased, the product pattern changed. The amount of $C_2F_2Cl_2$ formed after 60 min increased significantly (Figure 3). Less $CHFCl_2$ and more CH_2FCl and CH_3F were found, indicating that $CHFCl_2$ was subject to further reduction when the Ti(III)/$CFCl_3$ ratio was increased. This was confirmed in an experiment containing an 8-fold excess of reducing agent. The progress curves presented in Figure 4 demonstrate that $CFCl_3$ is successively dechlorinated to its lower homologues.

Slightly higher rates and an almost identical product pattern were observed when the reactions were run at pH 6.5 rather than at pH 7.5.

Reductive Dehalogenation of CF_2Cl_2 and CF_3Cl. Aquocobalamin was also found to catalyze the reduction of FREONs 12 and 13 to CO, albeit at much lower rates than the rate of FREON 11 reduction. The rates decreased in the series $CFCl_3$, CF_2Cl_2, and CF_3Cl. CF_4 was not reduced at measurable rates. The rate of fluoride release from CF_2Cl_2 almost equaled that of CF_2Cl_2 consumption (Figure 5). However, much less CO was formed than could be accounted for by the observed defluorination. No significant amounts of other organic products were detected.

Cobinamides and Methyl Corrinoids as Catalysts. In addition to aquocobalamin, cobinamides and methyl derivatives of these corrinoids were tested as catalysts in the reductive dehalogenation of $CFCl_3$. The results, summarized in Table

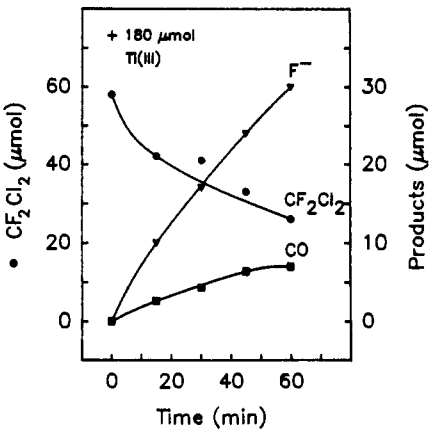


FIGURE 5: Corrinoid-catalyzed reductive dehalogenation of CF_2Cl_2 with titanium(III) citrate as electron donor. For assay conditions, see Figure 2. The 30-mL reaction mixture contained 60 μmol of CF_2Cl_2, 180 μmol of titanium(III) citrate, and 1.5 μmol of aquocobalamin in 0.2 M Tris-HCl, pH 7.5.

II, demonstrate that (cyanoaquo)cobinamide, (methylaquo)-cobinamide, and (cyanoaquo)trimethylbenzimidazoylcobamide were more effective than aquocobalamin. In the halomethylcobalamin series, trifluoromethylcobalamin was the most effective catalyst, followed by difluoromethyl- and monofluoromethylcobalamin.

Reductive Demethylation of Methyl- and Halomethylcobalamins. Incubation of methylcobalamin, monochloromethylcobalamin, and monofluoro-, difluoro-, and trifluoromethylcobalamin with titanium(III) citrate generated methane (Figure 6). The highest rate of CH_4 formation was observed from $ClCH_2$-Cbl. The rates decreased in the series

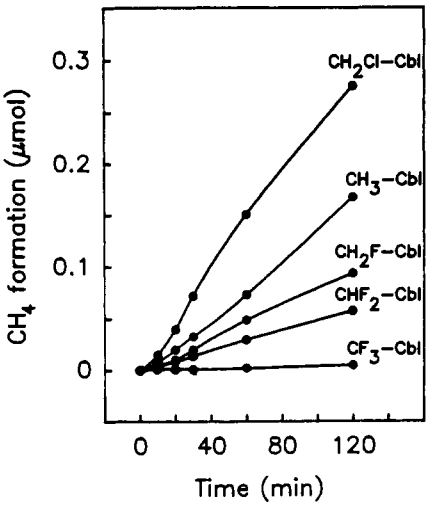


FIGURE 6: Methane formation from methyl corrinoids by reduction with titanium(III) citrate. Cbl represents cobalamin. The assays were performed in the dark at 30 °C in 8-mL serum bottles with N_2 as the gas phase. The 1-mL reaction mixture contained 1 μmol of the indicated methyl corrinoid and 25 μmol of titanium(III) citrate in 0.2 M Tris-HCl, pH 7.5. The reaction was started by the addition of the corrinoid. At the indicated intervals, 0.3-mL samples of the headspace were analyzed by gas chromatography.

$ClCH_2$-Cbl, CH_3-Cbl, FCH_2-Cbl, F_2CH-Cbl, and F_3C-Cbl.

Discussion

The results presented above demonstrate that the reductive dehalogenation of CCl_4, $CFCl_3$, and CF_2Cl_2, catalyzed by corrinoids, involves the intermediacy of dihalocarbenes. The evidence for chlorofluorocarbene as an intermediate in the reduction of $CFCl_3$ is 2-fold: first, CO was a major product derived from this FREON, and the rate and extent of CO formation paralleled that of F^- release; second, 1,2-dichloro-1,2-difluoroethene, the product of chlorofluorocarbene coupling, was formed in significant quantities.

$$CFCl_3 + 2[H] \rightarrow CFCl + 2HCl \quad (n)$$

$$CFCl + H_2O \rightarrow CO + HF + HCl \quad (o)$$

$$2CFCl \rightarrow C_2F_2Cl_2 \quad (p)$$

The formation of small amounts of formate is considered further evidence, because the reaction of CFCl with two molecules of H_2O is expected to yield formate.

$$CFCl + 2H_2O \rightarrow HCOOH + HF + HCl \quad (q)$$

CO formation from CF_2Cl_2 involves difluorocarbene as the intermediate. This carbene is known to be more stable than CClF but also hydrolyzes in aqueous solution.

$$CF_2Cl_2 + 2[H] \rightarrow CF_2 + 2HCl \quad (r)$$

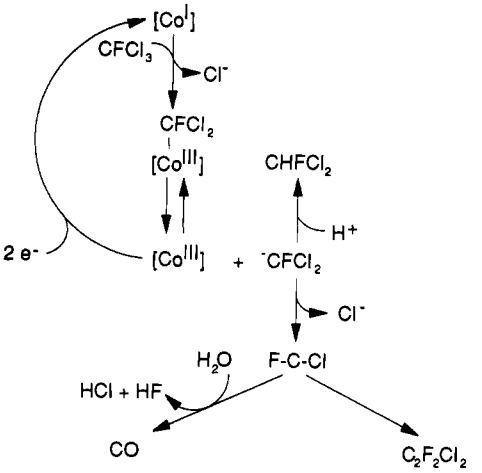
$$CF_2 + H_2O \rightarrow CO + 2HF \quad (s)$$

Reaction s is supported by the findings that CO formation and F^- release proceeded in parallel.

The reductive dehalogenation of CCl_4 to CO must also involve a dichlorocarbene that is expected to be more reactive than the chlorofluoro- or difluorocarbene.

The formation of highly reactive carbenes from CCl_4 and from FREONs 11 and 12 under reducing conditions may explain why these compounds are highly toxic for some anaerobic bacteria. These compounds may be considered mechanism-based inhibitors of the enzyme system catalyzing the reversible formation of acetyl-CoA in the acetyl-CoA/CO dehydrogenase pathway.

Scheme I



A possible mechanism for a two-electron reductive dehalogenation of $CFCl_3$ to CO and $CHFCl_2$ is outlined in Scheme I. Nucleophilic attack of a Co(I) corrinoid on $CFCl_3$ gives a monofluorodichloromethyl corrinoid that reversibly yields a monofluorodichloro carbanion and a Co(III) corrinoid. The monofluorodichloro carbanion is either protonated to $CHFCl_2$ or eliminates chloride ion to give chlorofluorocarbene. The latter hydrolyzes to CO, HF, and HCl or dimerizes to dichlorodifluoroethene.

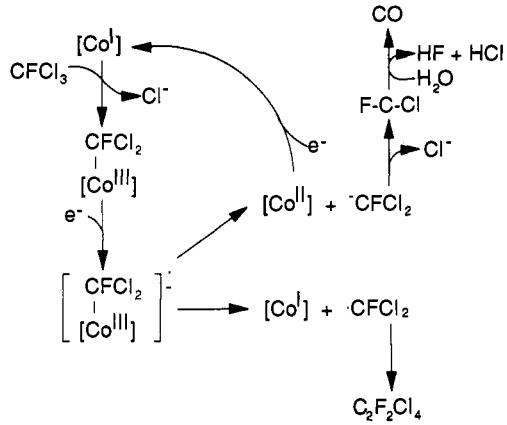
The corrinoid-catalyzed dehalogenation of CCl_4, $CFCl_3$, and CF_2Cl_2 showed very distinct product patterns. CCl_4 gave primarily reduction products in which the chlorine atoms are substituted by hydrogen ($CHCl_3$, CH_2Cl_2, and CH_3Cl), while less than 10% of the chlorocarbon was converted to CO (Figure 1). In contrast, the reductive dehalogenation of $CFCl_3$ gave predominantly CO and $C_2F_2Cl_2$, derived from chlorofluorocarbene, and only minor amounts of $CHFCl_2$, CH_2FCl, and CH_3F (Figure 2). The reductive dehalogenation of CF_2Cl_2 was much slower, and other than CO only traces of other volatile products were detected.

This type of product pattern is exactly what would be predicted from the well-established reactivity of the trihalomethyl carbanions (Hine, 1962; Chambers, 1973). Fluoro substituents, compared to the other halogens, destabilize the trihalomethyl carbanions, but they greatly stabilize the dihalocarbenes. Thus the trichloromethyl carbanion derived from carbon tetrachloride is more stable and is primarily protonated to chloroform. In contrast, dichlorofluoromethyl carbanion and chlorodifluoromethyl carbanion derived from $CFCl_3$ and CF_2Cl_2, respectively, decompose to chlorofluorocarbene and difluorocarbene much more readily. Hydrolysis of dihalocarbene yields carbon monoxide. Indeed, the chlorodifluoromethyl carbanion may be so unstable that difluorocarbene formation becomes concerted and the chlorodifluoromethyl carbanion stage is bypassed.

In this context, it should also be pointed out that all attempts to synthesize trichloromethyl- or dichloromethylcobalamins or cobinamides have failed, presumably because these compounds hydrolyze spontaneously, yielding $CHCl_3$ or CH_2Cl_2. The earlier reported syntheses of these corrinoids (Wood et al., 1968) could not be repeated.

In addition to 1,2-dichloro-1,2-difluoroethene, the product of chlorofluorocarbene coupling, traces of 1,1,2,2-tetrachloro-1,2-difluoroethane were also detected in the reductive dehalogenation of $CFCl_3$. Such a product would be expected from the coupling of dichlorofluoromethyl radicals. The reaction sequence outlined in Scheme II could account for the

Scheme II



generation of radical intermediates. One-electron reduction of the fluorodichloromethyl corrinoid would generate a fluorodichloromethyl corrinoid radical anion that could undergo either homolytic or heterolytic cleavage of the carbon–cobalt bond. Homolytic cleavage would yield a fluorodichloromethyl radical and a Co(I) corrinoid, while heterolytic cleavage would generate a fluorodichloromethyl anion and a Co(II) corrinoid. Such a mechanism has been suggested by Scheffold et al. (1987), who indicated that alkyl corrinoids with strong electron-withdrawing groups are readily reduced to the radical anions. It should be noted that both mechanisms yield trihalo carbanions, which could either lose a halide ion to form a dihalocarbene or accept a proton to generate trihalomethane.

As outlined in Table II, a wide variety of cobalamins and cobinamides are able to serve as catalysts in the reductive dehalogenation reactions. The cobinamides and (cyanoaquo)trimethylbenzimidazoylcobamide, which lack the 5,6-dimethylbenzimidazole ligand in the lower coordination position, were found to be the more effective catalysts.

However, it should also be noted that the catalytic efficiency of the alkylcobalamins is not identical but that it increased in the series $CH_3Cbl < FCH_2Cbl < F_2CHCbl < F_3CCbl$. These findings indicate that the carbon–cobalt bond of these cobalamins remained intact during the catalytic process. If the organometallic bond of these cobalamins had been cleaved, they should have behaved identically and in the same manner as aquocobalamin after one turnover. Earlier, we proposed a mechanism for the reductive dehalogenation of chlorinated C_1-hydrocarbons catalyzed by corrinoids that involved the intermediacy of *trans*-dialkylcobalt(III) complexes (Krone et al., 1989a). Our present observations also point to such a mechanism.

Halomethylcobalamins, possible intermediates in the reductive dehalogenation, were converted to methane under the experimental conditions (Figure 6). However, the introduction of fluorine atoms decreased the rate of methane formation. Indeed, CF_3Cbl, the most efficient catalyst for the reduction of $CFCl_3$, was reduced to CH_4 at marginally detectable rates.

As pointed out above, the reaction of cob(I)alamin with $CHFCl_2$ did yield FCH_2Cbl and not FClCHCbl, indicating that, under the reaction conditions, FClCHCbl is converted to FCH_2Cbl. Brown et al. (1984) and Tachkova et al. (1979) also showed that the alkylation of cob(I)alamin with FREONs, such as CF_3I and CHF_2Cl, under certain conditions yielded not only the fluoroalkylcobalamins but also their defluorinated derivatives.

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Localization in the Golgi Apparatus of Rat Liver UDP-Gal:Glucosylceramide β1→4Galactosyltransferase†

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ABSTRACT: The presence and subcellular localization of UDP-Gal:glucosylceramide β1→4galactosyltransferase (GalT-2) was investigated in rat liver. For this purpose, purified Golgi apparatus, endoplasmic reticulum, and plasma membrane fractions were prepared from the liver and used as the enzyme source for detecting GalT-2. A pure Golgi apparatus, highly enriched in many glycosyltransferases, was the only fraction where GalT-2 was measurable. The reaction product formation rate under appropriate assay conditions, which requires high detergent concentration and Mn^{2+}, was low but comparable with that of other glycosyltransferases. The product formation was stimulated by exogenously added acceptor GlcCer, donor UDP-Gal, and Golgi protein. The reaction product was a single spot that was identified by chromatographic behavior, sensitivity to β-galactosidase, and permethylation studies as Galβ1→4Glcβ1→1′Cer (lactosylceramide). A metabolic experiment, performed by determining the glycosphingolipids which became radioactive in the above subcellular fractions prepared from the liver of animals treated with glucose-labeled glucosylceramide, further indicated that the in vivo glycosylation of glucosylceramide takes place in the Golgi apparatus.

Glycosyltransferases involved in the elongation and termination of the oligosaccharide chain of glycosphingolipids and glycoproteins have been extensively studied, and much information is available now on their catalytic properties, specificity, and subcellular localization. For some of them, details are also known at the protein and gene level (Paulson & Colley, 1989).

Many results were also generated on the initial assembly of the oligosaccharide chain of Asn-linked glycoproteins (Hirschberg & Snider, 1987), and some reports also recently investigated the first biosynthetic steps occurring in the biosynthesis of the oligosaccharide chain of O-linked glycoproteins (Abeijon & Hirschberg, 1987; Piller et al., 1990). However, the activities involved in the initial glycosylation of glycosphingolipids were poorly studied. UDP-Gal:glucosylceramide (GlcCer)[1] β1→4galactosyltransferase, or GalT-2, is the enzyme activity responsible for the biosynthesis of LacCer, a common precursor of many acid and neutral glycosphingolipids. After its first discovery in the nervous system (Basu et al., 1968) and in the rat spleen (Hildebrand & Hauser, 1969), it was reported in other tissues such as bone marrow (Taki et al., 1982) and renal cells (Chatterjee & Castiglione, 1987). In rat liver, the presence of GalT-2 was suggested (Senn et al., 1983), but exogenous GlcCer-dependent formation of true LacCer could not be demonstrated in vitro (Walter et al., 1983). In vivo, the occurrence of glycosylation of exogenous GlcCer (Trinchera et al., 1990b) suggests that GalT-2 may be present in this tissue. Moreover, the subcellular localization of GalT-2 has not yet been investigated in any tissue or cultured cell, even though it is a central point, especially in light of the possible regulatory effect exerted by exogenous substances on its activity (Chatterjee et al., 1988).

In this paper, we attempted to identify and characterize GalT-2 in rat liver, with the aim of establishing its subcellular localization with respect to the other glycosyltransferase activities involved in glycosphingolipid biosynthesis. In addition, we wanted to determine within which subcellular fraction exogenous GlcCer undergoes glycosylation in vivo. For these purposes we tested galactosyltransferase activity toward exogenous GlcCer using different cellular subfractions as the enzyme source. We prepared enough reaction product, radiolabeled in two different positions of the molecule, in order to unambiguously establish its structure. Further, we determined the amount of glycosphingolipids which became radioactive in the above subcellular fractions prepared from the liver of animals treated with glucose-labeled GlcCer ([*Glc*-^{3}H]GlcCer) and sacrificed at two different times after the injection.

Experimental Procedures

Materials. GlcCer and LacCer were prepared by controlled acid hydrolysis (0.5 M HCl, 4 h, 80 °C) of a bovine brain ganglioside mixture (Ghidoni et al., 1980). They were sepa-

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[1] Abbreviations: HPTLC, high-performance thin-layer chromatography; GLC, gas–liquid chromatography. Gangliosides and glycosphingolipids are coded according to the nomenclature of Svennerholm (1964) and the *IUPAC–IUB Recommendations* (1977). Designation of glycosphingolipid glycosyltransferases is according to Basu et al. (1987) with the reported extension (Trinchera et al., 1990a).

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